

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of	:	Customer Number: 20277
	:	
Wayne D. COMPER	:	Confirmation Number: 2638
	:	
Serial No.: 09/893,346	:	Group Art Unit: 1648
	:	
Filed: June 28, 2001	:	Examiner: S. S. Brown
	:	
For: METHOD FOR KIDNEY DISEASE TREATMENT BY DRUG INTERVENTION	:	

**DECLARATION UNDER 37 C.F.R § 1.132**

Dear Sir:

I, Wayne Comper, hereby declare and say as follows:

1. That I am an Australian citizen , residing in New York, New York, and am the inventor of the invention described and claimed in application Serial No. 10/391,202. My curriculum vitae is attached as Exhibit A.

2. I have read and am familiar with the disclosure and pending claims of the above-captioned Application, as well as the issues raised in the pending Office Action dated June 30, 2004.

3. I declare and state that:

Until recently it HSA been assumed that native albumin which is filtered from the blood by the kidney is excreted in an unmodified, native form (see for example Myers BD, Winetz JA, Chui F, Michaels AS. *Mechanism of proteinuria in diabetic nephropathy; a study of glomerular barrier function*. Kidney Int 21: 633-641 1982) That is, is HSA been assumed that the albumin present in urine is immunologically the same as that present in plasma.

Antibodies generated to native serum albumin have been widely used commercially in urine immunochemical-based assays to detect urinary albumin. These antibodies measure generally the same form of albumin as demonstrated in Proficiency

Testing Service studies carried out by the American Association of Bioanalysts (AAB)([www.aab.org/pts/ch3q03/microalb.pdf](http://www.aab.org/pts/ch3q03/microalb.pdf)). The AAB have analyzed essentially all the available commercial immunoassays of which there are 13. Although there is some interassay variation they all give similar results. I have never seen any publication where antibodies have been raised to urinary albumin, let alone their use in measuring urinary albumin. Accordingly, one of ordinary skill in the art would understand conventional antibodies to refer to those antibodies generated to native serum protein, like albumin, and that immunoassays employing conventional antibodies are conventional immunoassays.

More recently my studies have demonstrated that human urinary albumin exists in two forms: one form that is immuno-reactive having the native structure of plasma albumin and a second form that is physicochemically similar to native albumin except that it is immuno-nonreactive. This immuno-nonreactive albumin HSA been modified during renal passage and is not measured by conventional immunoassays (Comper WD, US 6589748, Comper U.S. 6447989; Comper WD, Osicka TM, Jerums G. *High prevalence of immuno-unreactive intact albumin in urine of diabetic patients*. Am J Kid Dis 41: 336-342, 2003; Comper WD, Jerums G, Osicka TM. *Deficiency in the detection of microalbuminuria by urinary dipstick in diabetic patients*. Diabetes Care 26:c3195-3196, 2003; Comper WD, Osicka TM, Clark M, MacIsaac RJ, Jerums G. *Earlier Detection of microalbuminuria in diabetic patients using a new urinary albumin assay*. Kidney Int. 65:1850-1855, 2004; Osicka TM, MacIsaac RJ, Jerums G, Comper WD. *High prevalence of immunounreactive albumin in urine from diabetic patients with a low glomerular filtration rate and normoalbuminuria*. Diabetes Care 27:1515, 2004; Comper WD, Jerums G, Osicka TM. *Differences in urinary albumin detected by four immunoassays and high performance liquid chromatography*. Clin Biochem 37: 105-111, 2004; Osicka TM, Comper WD. *Albumin-like material in urine*. Kidney Int 2004 (in press)).

We have demonstrated that the amount of albumin in urine detected by HPLC is much higher than that detected by commercially available immunoassays. The reason for the discrepancy is that HPLC detects both immuno-reactive and immuno-nonreactive albumin, while commercial or conventional immunoassays detect only immuno-reactive

albumin. The immunoassays tested include an in-house radioimmunoassay employed at the Austin & Repatriation Medical Centre, Heidelberg, Melbourne Australia, immunonephelometry (Beckman Array using reagents from Beckman Diagnostics) and two different methods of immunoturbidimetry (Dade-Behring Turbitimer using reagents from Dade-Behring, and Dade-Behring Dimension RxL Chemistry Analyser using reagents from Diasorin). Analysis of both immuno-reactive and immuno-nonreactive intact albumin is the basis of the Accumin™ HPLC assay.

The FDA HSA recognized the differences between the HPLC assay and conventional immunoassays that use antibodies directed to native serum albumin. The FDA acknowledges that the antibodies to serum albumin do not detect the modified albumin material in urine. FDA labelling of the Accumin™ assay states the following: *“Note: The Accumin™ HPLC assay detects albumin based on size exclusion chromatography calibrated by human serum albumin standard. The size-exclusion HPLC technology employed by the Accumin™ HPLC assay permits a direct measurement of albumin, regardless of the reactivity potential of the protein with antibodies. Since immunoassays (and dye binding assays) may not detect all of the intact albumin in urine samples it is expected that the HPLC technology employed by the Accumin™ HPLC assay will, depending on the specimen, report greater urinary albumin values when compared to immunochemical urinary albumin test systems and dipstick system. This trend HSA been observed at lower excretion rates, sometimes up to 100 µg/min in urine samples from diabetic patients.*

4. We have tested monoclonal antibodies that are commercially available from the following companies to determine whether these antibodies detect modified albumin:

Maine Biotechnology Services  
Monoclonal MAB587P  
Antigen: Human Serum Albumin

Scripps Laboratories  
Monoclonals CA002, CA003, CA005 and CA006  
Antigen: Human Serum (or Plasma) Albumin

Fitzgerald Industries  
Monoclonal M57249

Antigen: Human Albumin (source unspecified)

Biodesign International

Monoclonals 3002, 1C8, 15C7, 3001, 3004, 3003, 6B11, 1A9 and 3005

Antigen: Human Serum Albumin

BiosPacific

Monoclonals 0228P, 1228P and 2228P

Antigen: Unspecified

We also tested thirty different, commercially available polyclonal rabbit anti-HSA antibodies.

5. The following tests were carried out under my supervision to determine whether anti-human serum albumin (HSA) antibodies (polyclonal and monoclonal) detect or cross-react with modified urinary albumin :

(1) ELISA screening of anti-human albumin sera was carried out in 96-well microtiter plates in which antigen (HSA) (50  $\mu$ l/well) had been incubated at 4°C overnight, and then washed. The wells were then blocked with 1% gelatin in MTPBS, treated with buffer and rinsed three times with PBS. Sera was added at appropriate dilutions and incubated at room temperature in a humid box for one hour. Sera was screened in duplicate or triplicate using dilutions of 1:50 to 1:50,000. After incubation, secondary antibody (anti-mouse Ig, affinity isolated, HRP conjugated (Silenus) or anti-mouse IgG Fc  $\gamma$ -specific, HRP conjugated (Jackson Labs) was added at 50  $\mu$ l/well in buffer and incubated for one hour at room temperature. The wells were then washed, substrate was added (50  $\mu$ l/well) and color development was allowed to take place. Plates were read after 15 minutes at 440-450 nm on an ELISA plate reader.

(2) ELISA screening of commercially obtained albumin monoclonal antibodies was carried out in 96-well microtiter plates in which 200  $\mu$ l/well of diluted target antigen (HSA or modified urinary albumin ("ghAlb")) was diluted to a concentration of 10  $\mu$ g/ml in 0.1 M carbonate, pH 9.5 and incubated overnight at 4°C overnight. The plates were then washed to remove unbound materials and the wells were blocked with 300  $\mu$ l/well 1% casein in 0.1 M carbonate, pH 9.5 and incubated at 37°C for one hour. Incubation was followed by a wash pHSAe and addition of 100  $\mu$ l/well of each mouse antiserum or

cell supernatant plus 100 µl of assay buffer (phosphate buffered saline with 0.1% gelatin) to appropriate wells. “Blank” antisera were substituted with 100 µl of assay buffer for nonspecific binding determination. The plates were agitated to ensure antibody-antigen mixture and incubated at 37 °C for one hour. After incubation the plates were washed and 200 µl of AP-linked anti-mouse IgG (Sigma, St. Louis, MO) diluted 1:25,000 in assay buffer was added to each well and incubated at 37 °C for another hour. After incubation the plates were washed and 200 µl of p-nitrophenyl phosphate (Sigma, St. Louis, MO) reconstituted in 1 mg/ml in alkaline phosphate substrate buffer was added to each well and allowed to develop for ~30 minutes. Plates were read at 405 nm on a multiplate spectrophotometer.

The data obtained in these assays are shown in the three tables that are provided on separate pages attached hereto. Human serum albumin was used as a positive control in these studies.

6. The ELISA screening assays provide qualitative information concerning cross-reactivity of the tested antibodies. Based on the results obtained, we determined that a reactivity greater than 10% is significant, while percentages less than 10% can be due to non-specific interactions, minor contaminations of the screening antigen preparations with either the specific antigen (HSA) or an interfering substance, (for example IgG contamination results in elevated background when using an anti-rabbit IgG secondary antibody), or a lack of sufficient specificity in the screened anti-HSA preparation (as with unpurified antisera).

Using a cut-off of >10% reactivity, we found that modified albumin preparation, M7#3, which was used for screening polyclonal antibodies, shows the only significant reactivity for a large number of commercially available anti-HSA antisera. Since this preparation was derived from an identical pool source as the M7#1 and M7#2 preparations, this indicates that a specific contaminant was introduced into M7#3 during its purification. Further investigation HSA shown that this contaminant is likely to be unmodified HSA which was not entirely removed by affinity purification.

All other modified albumin preparations showed less than 10% relative HSA reactivity for all monoclonal and polyclonal anti-HSA antisera with one exception.

The Cambio (Cambridge, UK) rabbit anti-HSA antiserum showed a minimal amount of reactivity for Fraction V HSA and slightly elevated reactivity for the modified albumin preparations from IBA (Branford, CT). There are a number of factors that may have influenced this result. For example, the antiserum is provided undialysed and is not affinity purified. This raises questions about its specificity and the possible contamination of the preparation with cross-reactive components. Further, this preparation was made by immunizing host rabbits with a highly purified, crystalline fatty-acid free HSA preparation. The anti-serum is specific for fatty-acid free HSA and HSA little cross-reactivity with traditionally antigenic HSA preparations.

Further, Cambio rabbit anti-HSA polyclonal antibody showed a slight level of reactivity with the IBA-generated modified albumin, but showed no reactivity with Ausam-generated modified albumin, suggesting the existence of a specific component or contaminant in the IBA preparation. The contaminant or component in the IBA preparation may be fatty-acid free HSA, but might also be a cross-reactive contaminant such as human IgG non-specifically bound by the "raw" antiserum.

Overall, the results obtained from the ELISA tests of the forty eight commercially available anti-albumin antibodies demonstrate that antibodies made to serum albumin do not cross react with modified urinary albumin.

7. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

4<sup>th</sup> October 2004

Date



Wayne Comper

<b>Plate A</b>							
<b>Monoclonal mouse Ab preps</b>							
<b>Blank</b>		0.162	0.149	0.147	0.149	0.148	0.147
<b>MAB 587P</b>	<b>Marine Bio</b>	0.171	0.152	0.281	0.281	0.149	0.149
<b>cloneCA002</b>	<b>Scripps</b>	0.149	0.152	0.568	0.605	0.205	0.204
<b>cloneCA003</b>	<b>Scripps</b>	0.151	0.151	0.379	0.421	0.149	0.148
<b>cloneCA005</b>	<b>Scripps</b>	0.150	0.151	0.595	0.597	0.191	0.185
<b>cloneCA006</b>	<b>Scripps</b>	0.152	0.157	0.688	0.760	0.233	0.238
<b>cloneM57249</b>	<b>Scripps</b>	0.154	0.149	0.550	0.548	0.152	0.159
<b>Blank</b>		0.149	0.151	0.151	0.151	0.149	0.151
<b>Averaged absorbances multiplied by 1000:</b>							
		<b>Blank</b>		<b>HSA</b>		<b>ghAlb</b>	
<b>Blank</b>		156		148		148	
<b>MAB 587P</b>	<b>Marine Bio</b>	162		281		149	
<b>cloneCA002</b>	<b>Scripps</b>	151		587		205	
<b>cloneCA003</b>	<b>Scripps</b>	151		400		149	
<b>cloneCA005</b>	<b>Scripps</b>	151		596		188	
<b>cloneCA006</b>	<b>Scripps</b>	155		724		236	
<b>cloneM57249</b>	<b>Scripps</b>	152		549		156	
<b>Blank</b>		150		151		150	
<b>Blank subtracted:</b>							
				<b>HSA</b>		<b>ghAlb</b>	
<b>Blank</b>				(8)		(8)	
<b>MAB 587P</b>	<b>Marine Bio</b>			120		(13)	
<b>cloneCA002</b>	<b>Scripps</b>			436		54	
<b>cloneCA003</b>	<b>Scripps</b>			249		(3)	
<b>cloneCA005</b>	<b>Scripps</b>			446		38	
<b>cloneCA006</b>	<b>Scripps</b>			570		81	
<b>cloneM57249</b>	<b>Scripps</b>			398		4	
<b>Blank</b>				1		0	
<b>%Proportional reactivity (ghAlb/HSA)</b>							
						<b>%HSA</b>	
<b>Blank</b>							
<b>MAB 587P</b>	<b>Marine Bio</b>					(10)	
<b>cloneCA002</b>	<b>Scripps</b>					12	
<b>cloneCA003</b>	<b>Scripps</b>					(1)	
<b>cloneCA005</b>	<b>Scripps</b>					8	
<b>cloneCA006</b>	<b>Scripps</b>					14	
<b>cloneM57249</b>	<b>Scripps</b>					1	

<b>Plate A</b>							
<b>Monoclonal mouse Ab preps</b>							
H45300M	Biodesign	0.152	0.160	0.548	0.562	0.191	0.192
H45700M	Biodesign	0.149	0.151	0.601	0.595	0.177	0.180
H45705M	Biodesign	0.155	0.150	0.980	0.960	0.214	0.222
H45304M	Biodesign	0.149	0.149	0.710	0.649	0.229	0.236
H45965M	Biodesign	0.147	0.146	0.501	0.545	0.161	0.164
H86910M	Biodesign	0.148	0.152	1.196	1.182	0.236	0.261
H86424M	Biodesign	0.151	0.149	0.623	0.620	0.180	0.176
Blank		0.148	0.153	0.148	0.149	0.149	0.150
<b>Averaged absorbances multiplied by 1000:</b>							
		<b>Blank</b>		<b>HSA</b>		<b>ghAlb</b>	
H45300M	Biodesign	156		555		192	
H45700M	Biodesign	150		598		179	
H45705M	Biodesign	153		970		218	
H45304M	Biodesign	149		680		233	
H45965M	Biodesign	147		523		163	
H86910M	Biodesign	150		1189		249	
H86424M	Biodesign	150		622		178	
Blank		151		149		150	
<b>Blank subtracted:</b>							
				<b>HSA</b>		<b>ghAlb</b>	
H45300M	Biodesign			399		36	
H45700M	Biodesign			448		29	
H45705M	Biodesign			818		66	
H45304M	Biodesign			531		84	
H45965M	Biodesign			377		16	
H86910M	Biodesign			1039		99	
H86424M	Biodesign			472		28	
Blank				(2)		(1)	
<b>%Proportional reactivity (ghAlb/HSA)</b>							
						<b>%HSA</b>	
H45300M	Biodesign					9	
H45700M	Biodesign					6	
H45705M	Biodesign					8	
H45304M	Biodesign					16	
H45965M	Biodesign					4	
H86910M	Biodesign					9	
H86424M	Biodesign					6	



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<b>Plate B</b>							
<b>Monoclonal mouse Ab preps</b>							
<b>Blank</b>		0.151	0.148	0.152	0.149	0.159	0.163
<b>H86157M</b>	<b>Biodesign</b>	0.172	0.152	0.579	0.577	0.158	0.157
<b>H86611M</b>	<b>Biodesign</b>	0.152	0.151	0.976	0.980	0.191	0.210
<b>A15370228P</b>	<b>Biopacific</b>	0.151	0.155	0.611	0.585	0.176	0.174
<b>A15371228P</b>	<b>Biopacific</b>	0.171	0.150	0.627	0.621	0.172	0.164
<b>A15372228P</b>	<b>Biopacific</b>	0.161	0.152	1.206	1.225	0.216	0.215
<b>A15373228P</b>	<b>Biopacific</b>	0.151	0.155	1.074	1.037	0.211	0.215
<b>Blank</b>		0.154	0.158	0.151	0.158	0.148	0.153
<b>Averaged absorbances multiplied by 1000:</b>							
		<b>Blank</b>		<b>HSA</b>		<b>ghAlb</b>	
<b>Blank</b>		150		151		161	
<b>H86157M</b>	<b>Biodesign</b>	162		578		158	
<b>H86611M</b>	<b>Biodesign</b>	152		978		201	
<b>A15370228P</b>	<b>Biopacific</b>	153		598		175	
<b>A15371228P</b>	<b>Biopacific</b>	161		624		168	
<b>A15372228P</b>	<b>Biopacific</b>	157		1216		216	
<b>A15373228P</b>	<b>Biopacific</b>	153		1056		213	
<b>Blank</b>		156		155		151	
<b>Blank subtracted:</b>							
				<b>HSA</b>		<b>ghAlb</b>	
<b>Blank</b>				1		12	
<b>H86157M</b>	<b>Biodesign</b>			416		(4)	
<b>H86611M</b>	<b>Biodesign</b>			827		49	
<b>A15370228P</b>	<b>Biopacific</b>			445		22	
<b>A15371228P</b>	<b>Biopacific</b>			464		7	
<b>A15372228P</b>	<b>Biopacific</b>			1059		59	
<b>A15373228P</b>	<b>Biopacific</b>			903		60	
<b>Blank</b>				(2)		(6)	
<b>%Proportional reactivity (ghAlb/HSA)</b>							
						<b>%HSA</b>	
<b>Blank</b>							
<b>H86157M</b>	<b>Biodesign</b>					(1)	
<b>H86611M</b>	<b>Biodesign</b>					6	
<b>A15370228P</b>	<b>Biopacific</b>					5	
<b>A15371228P</b>	<b>Biopacific</b>					2	
<b>A15372228P</b>	<b>Biopacific</b>					6	
<b>A15373228P</b>	<b>Biopacific</b>					7	

<b>Plate B</b>						
<b>Polyclonal rabbit Ab preps</b>						
	0.177	0.179	0.376	0.394	0.313	0.322
	0.161	0.163	0.345	0.323	0.307	0.309
	0.152	0.152	0.346	0.332	0.339	0.334
	0.156	0.150	0.333	0.348	0.327	0.332
	0.152	0.153	0.310	0.334	0.340	0.348
	0.158	0.157	0.307	0.313	0.333	0.345
	0.151	0.151	0.158	0.149	0.266	0.267
	0.153	0.153	0.150	0.149	0.256	0.257
<b>Averaged absorbances multiplied by 1000:</b>						
	<b>Blank</b>		<b>HSA</b>		<b>ghAlb</b>	
<b>anti-HSA</b>	178		385		318	
<b>A751</b>	162		334		308	
<b>A752</b>	152		339		337	
<b>241</b>	153		341		330	
<b>242</b>	153		322		344	
<b>244</b>	158		310		339	
<b>Blank</b>	151		154		267	
<b>Blank</b>	153		150		257	
<b>Blank subtracted:</b>						
			<b>HSA</b>		<b>ghAlb</b>	
<b>anti-HSA</b>			207		51	
<b>A751</b>			172		42	
<b>A752</b>			187		70	
<b>241</b>			188		63	
<b>242</b>			170		78	
<b>244</b>			153		72	
<b>Blank</b>			3		0	
<b>Blank</b>			(4)		(10)	
<b>%Proportional reactivity (ghAlb/HSA)</b>						
					<b>%HSA</b>	
<b>anti-HSA</b>					25	
<b>A751</b>					24	
<b>A752</b>					37	
<b>241</b>					34	
<b>242</b>					46	
<b>244</b>					48	
<b>Blank</b>					0	

[illegible]

# Polyclonal anti-HSA antisera screening vs ghAlb

HSA	ghAlb Preparation					
	IBA 1+2	IBA 3	Grydzyn	M7 #1	M7 #2	M7 #3
Accurate (gt)	0.439					
Bethyl (gt)	0.064	0.158	0.135	0.062	0.099	0.042
Calbiochem (gt)	0.466			0.048	0.053	0.064
Cortex (gt)	0.616					
Fitzgerald (gt)	0.838		0.037	0.052		0.052
ICN (gt)	0.733	0.049	0.044			0.062
RDI (gt)	0.686			0.048	0.036	0.046
Serotec (gt)	0.702		0.032		0.041	0.064
Midland (gt)	0.212					
Accurate (sh)	0.238					
Bethyl (sh)	0.675					
Calbiochem (sh)	0.771					
Cortex (sh)	2.197		0.029			0.129
Fitzgerald (sh)	2.338		0.231	0.211	0.082	0.963
ICN (sh)	1.907		0.039			0.158
RDI (sh)	2.418		0.229	0.287	0.137	0.777
Serotec (sh)	2.172					
Midland (sh)	0.094					
Accurate Chem (sh)	0.578	0.096	0.046		0.078	0.233
Bethyl (sh)	0.542				0.062	0.146
Calbiochem (sh)	0.513					0.131
Cortex (sh)	0.447					0.098
Fitzgerald (sh)	0.414					0.060
ICN (sh)	0.496					0.125
RDI (sh)	0.486					0.135
Serotec (sh)	0.464					0.131
Midland (sh)	0.545					
Accurate Chem (sh)	0.557					
Bethyl (sh)	0.615					
Calbiochem (sh)	0.564					
Cortex (sh)						
Fitzgerald (sh)						
ICN (sh)						
RDI (sh)						
Serotec (sh)						
Midland (sh)						
Accurate Chem (sh)						
Bethyl (sh)						
Calbiochem (sh)						
Cortex (sh)						
Fitzgerald (sh)						
ICN (sh)						
RDI (sh)						
Serotec (sh)						
Midland (sh)						
Accurate Chem (sh)						
Bethyl (sh)						
Calbiochem (sh)						
Cortex (sh)						
Fitzgerald (sh)						
ICN (sh)						
RDI (sh)						
Serotec (sh)						
Midland (sh)						
Accurate Chem (sh)						
Bethyl (sh)						
Calbiochem (sh)						
Cortex (sh)						
Fitzgerald (sh)						
ICN (sh)						
RDI (sh)						
Serotec (sh)						
Midland (sh)						
Accurate Chem (sh)						
Bethyl (sh)						
Calbiochem (sh)						
Cortex (sh)						
Fitzgerald (sh)						
ICN (sh)						
RDI (sh)						
Serotec (sh)						
Midland (sh)						
Accurate Chem (sh)						
Bethyl (sh)						
Calbiochem (sh)						
Cortex (sh)						
Fitzgerald (sh)						
ICN (sh)						
RDI (sh)						
Serotec (sh)						
Midland (sh)						
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Bethyl (sh)						
Calbiochem (sh)						
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ICN (sh)						
RDI (sh)						
Serotec (sh)						
Midland (sh)						
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Cortex (sh)						
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ICN (sh)						
RDI (sh)						
Serotec (sh)						
Midland (sh)						
Accurate Chem (sh)						
Bethyl (sh)						
Calbiochem (sh)						
Cortex (sh)						
Fitzgerald (sh)						
ICN (sh)						
RDI (sh)						
Serotec (sh)						
Midland (sh)						
Accurate Chem (sh)						
Bethyl (sh)						
Calbiochem (sh)						
Cortex (sh)						
Fitzgerald (sh)						
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RDI (sh)						
Serotec (sh)						
Midland (sh)						
Accurate Chem (sh)						
Bethyl (sh)						
Calbiochem (sh)						
Cortex (sh)						
Fitzgerald (sh)						
ICN (sh)						
RDI (sh)						
Serotec (sh)						
Midland (sh)						
Accurate Chem (sh)						
Bethyl (sh)						
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<b>Fitzgerald (rabbit):</b>	Chromatographically purified IgG in PBS. Raised :
<b>Cambio (rabbit):</b>	Rabbit serum (undialysed), not affinity purified. Ra
<b>Kent (goat):</b>	Monospecific serum (undialysed)

The relatively high backgrounds of the Grydzyn, M7#1, M7#2 and M7#3 preps when testing the rabbit anti-HSA antisera is due to a small but significant rabbit IgG contamination of these preps.

The presence of significant reactivity between the M7#3 ghAlb prep and the majority of goat anti-HSA antisera is confusing. The prep was derived from the same urine pool as M7#1 and M7#2 which are not well detected by these same antisera. It is possible that the M7#3 prep has been contaminated with a trace amount of HSA during its HPLC purification, although the general inability of sheep- or rabbit- anti-HSA antisera to detect such a difference disputes this.

All cross-reactive antisera are not documented to have been affinity purified. This leads to the possibility that the antisera may contain antibodies to other proteins. For instance, if the immunising material contained trace mounts of IgG or Trf, any anti-IgG or anti-Trf antibodies formed would not be removed from the sera. Both IgG and Trf are known minor contaminants of the ghAlb preps.

The Cambio (rb) antiserum is unusual in that it is only very weakly reactive for HSA, its specific antigen. This antibody has a stronger reactivity for some component of the IBA preps than for HSA. Since this antibody was raised against a highly purified fatty-acid free HSA, it is possible that it is unable to bind natively occurring HSA.

		% of HSA reactivity				
IBA 1+2	IBA 3	Grydzyn	M7 #1	M7 #2	M7 #3	
0.00	0.00	0.00	0.00	0.00	0.00	
246.88	210.94	96.87	154.69	145.81	65.63	
0.00	0.00	10.30	11.37	13.73	10.09	
0.00	0.00	0.00	0.00	0.00	0.00	
0.00	4.42	6.21	0.00	6.21	7.40	
6.68	6.00	0.00	0.00	0.00	0.00	
0.00	0.00	7.00	5.25	6.71	7.00	
0.00	4.56	0.00	0.00	5.84	9.12	
0.00	0.00	0.00	0.00	0.00	0.00	
0.00	0.00	0.00	0.00	0.00	0.00	
0.00	0.00	0.00	0.00	0.00	0.00	
0.00	0.00	0.00	0.00	0.00	0.00	
0.00	1.32	0.00	0.00	0.00	5.87	
0.00	9.88	9.02	3.51	1.53	41.19	
0.00	2.05	0.00	0.00	0.00	8.29	
0.00	9.47	11.87	5.67	4.76	32.13	
0.00	0.00		0.00	0.00	0.00	
0.00	0.00		0.00	0.00	0.00	
16.61	7.93		13.49	10.73	40.31	
0.00	0.00		0.00	0.00	26.94	
0.00	0.00		0.00	0.00	25.54	
0.00	0.00		0.00	0.00	21.92	
0.00	0.00		0.00	0.00	14.49	
0.00	0.00		0.00	0.00	25.20	
0.00	0.00		0.00	0.00	27.78	
0.00	0.00		0.00	0.00	28.23	
0.00	0.00	0.00	0.00	0.00	0.00	
0.00	0.00	0.00	0.00	0.00	0.00	
0.00	0.00	0.00	0.00	0.00	0.00	
0.00	0.00	0.00	0.00	0.00	0.00	

0.00	8.30	8.40	3.30	0.00	38.30	
0.00	5.60	9.00	3.18	0.00	27.66	
311.76	253.82	0.00	0.00	0.00	0.00	
0.00	0.00	0.00	0.00	0.00	0.00	
6.96	3.80	0.00	0.00	0.00	0.00	

ghAlb preps

† some ghAlb preps

significant detection of M7#3 prep

significant detection of M7#3 prep

mal detection of HSA(!)

‡ not affinity purified

d against affinity purified human albumin

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against human albumin.  
raised against highly purified crystalline FA-free HSA



# Characterization of Immunochemically Nonreactive Urinary Albumin

TANYA M. OSICKA<sup>1,2</sup> and WAYNE D. COMPER<sup>1,2\*</sup>

**Background:** Conventional immunoassays underestimate the urinary albumin concentration because intact albumin in urine exists in two forms, immunoreactive and immunochemically nonreactive.

**Methods:** Urinary albumin concentration measured by HPLC (which measures total albumin, i.e., the sum of immunoreactive albumin + immunochemically nonreactive albumin) or RIA was compared with densitometric analysis of albumin bands in diabetic urine samples separated by either native polyacrylamide gel electrophoresis (PAGE) or reducing sodium dodecyl sulfate (SDS)-PAGE. Immunochemically nonreactive albumin was also isolated from diabetic urine (relative amount detected, 70–80% of the expected) and was tested for contamination by common urinary proteins by native PAGE, ELISA, and capillary electrophoresis.

**Results:** Urinary albumin concentrations measured by native PAGE and HPLC were better correlated ( $r^2 = 0.83$ ) than concentrations measured by native PAGE and RIA ( $r^2 = 0.62$ ) because under native conditions both native PAGE and HPLC detect total albumin and not only the immunoreactive albumin alone that is measured by RIA. Urinary albumin concentrations measured by reducing SDS-PAGE and RIA were better correlated ( $r^2 = 0.84$ ) than concentrations measured by reducing SDS-PAGE and HPLC ( $r^2 = 0.65$ ) because under reducing conditions immunochemically nonreactive albumin is unstable and fragments into many smaller peptides. The partially purified preparation was found to contain <1% contamination by common urinary proteins and is stable to freezing and frequent freeze/thaw cycles.

**Conclusions:** The results are consistent with the interpretation that immunochemically nonreactive albumin

has a limited number of polypeptide chain scissions and is held together by noncovalent intrachain bonding and disulfide bonds. Detection of this molecule is likely to be of clinical importance in diagnosing kidney disease as well as cardiovascular disease.

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Measurement of albumin ( $M_r$  66 000) in urine is complicated because urinary albumin may exist in multiple forms. This has been highlighted by the discovery that diabetic rats excrete increased quantities of intact albumin during the development of albuminuria that are undetectable by conventional immunoassays (immunochemically nonreactive albumin) (1). Immunochemically nonreactive albumin has also been found in urine from diabetic patients analyzed by HPLC, which is able to measure both immunoreactive albumin and immunochemically nonreactive albumin (total albumin) (2). In addition, we have found in patients with type 1 and type 2 diabetes that measurement of total albumin by HPLC can predict the onset of persistent albuminuria 3.9 and 2.4 years earlier, respectively, than can measurement of immunoreactive albumin alone by conventional RIA (3). These results not only identify that progression from normo- to microalbuminuria in diabetic patients is associated with an increase in urinary immunochemically nonreactive albumin, but raises the possibility that measurement of total albumin may allow earlier detection of progression to kidney disease (3). Similar results have also been found in peripheral vascular disease (4).

The aim of the present study was to further characterize the immunochemically nonreactive albumin species present in human diabetic urine. We compared quantitative analysis of total urinary albumin measured by HPLC or immunoreactive albumin measured by RIA with densitometric analysis of the albumin band in urine samples separated by native polyacrylamide gel electrophoresis (PAGE)<sup>3</sup> and reducing sodium dodecyl sulfate (SDS)-

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<sup>3</sup> Nonstandard abbreviations: PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; HSA, human serum albumin; and LC/MS/MS, liquid chromatography–tandem mass spectrometry.

PAGE. The purified immunochemically nonreactive albumin preparation was also analyzed for contaminants by HPLC, ELISA, and capillary electrophoresis.

### Materials and Methods

#### CHARACTERIZATION OF IMMUNOCHEMICALLY NONREACTIVE ALBUMIN

**PAGE.** Urines collected from diabetic patients ( $n = 32$ ) over 24 h (stored at  $-20^{\circ}\text{C}$ ) were analyzed by native PAGE and reducing SDS-PAGE. The total albumin content of the samples was determined by HPLC (see below). Approximately  $0.3\text{ }\mu\text{g}$  of protein was separated by electrophoresis in 12.5% gels at 200 V for 2 h (Bio-Rad Laboratories) and then silver-stained using Proteosilver staining reagents (Sigma-Aldrich Co). Variation between gels was corrected through the use of a standard urine included in each gel. Albumin band thickness was quantified by computer-aided densitometry (MCID software, Ver. 7.0; Imaging Research Inc.). The background density of the gel was subtracted from each band, and results were expressed as relative absorbance. The relative absorbance determined for the albumin band in each urine was quantified by use of an albumin calibrator run on each gel, and this was compared with the urinary albumin concentrations measured by HPLC analysis, which detects total albumin (i.e., the sum of immunoreactive albumin + immunochemically nonreactive albumin) and RIA, which detects immunoreactive albumin only. Densitometry quantified in this manner was found to be linear for human serum albumin (HSA) calibrator solutions up to  $100\text{ mg/L}$ . In addition, bands excised from the native gels were sequenced by liquid chromatography–tandem mass spectrometry (LC/MS/MS) by the Australian Proteome Analysis Facility (North Ryde, Sydney, Australia).

**HPLC.** Another group of fresh (unfrozen) diabetic urines were also analyzed by HPLC, and then reanalyzed after freezing at  $-20$  and  $-80^{\circ}\text{C}$  for 24 h, 1 week, and 2 months to determine whether freezing had an effect on urine composition. In addition, urines containing large amounts of immunochemically nonreactive albumin were analyzed after multiple (six) freeze/thaw cycles.

#### ALBUMIN ASSAYS

**Immunoassays.** Immunoreactive albumin was measured by either a double-antibody RIA (in-house) or by immunoturbidimetry on a Dade-Behring Turbitimer with reagents supplied by Dade-Behring Marburg GmbH as described previously (2, 3).

**HPLC analysis.** Total urinary albumin (immunoreactive albumin plus immunochemically nonreactive albumin) was determined by analyzing urine samples using an Agilent 1100 HPLC system (Agilent Technologies) (2, 3). Aliquots of urine ( $25\text{ }\mu\text{L}$ ) were injected on a Zorbax GF-250 analytical column [ $9.4\text{ mm (i.d.)} \times 250\text{ mm}$ ]. The mobile phase was phosphate-buffered saline, pH 7.0, at a

flow rate of  $1\text{--}2\text{ mL/min}$ . The urinary albumin peak was identified to within  $\pm 2\%$  of the elution time of the monomeric albumin calibrator. The intraassay CVs were 5.6% and 6.0% at concentrations of  $44.7\text{ mg/L}$  and  $141\text{ mg/L}$ , respectively, the interassay CV was 2.4% at  $95.9\text{ mg/L}$ , and the detection limit was  $2\text{ mg/L}$ .

#### ISOLATION OF IMMUNOCHEMICALLY NONREACTIVE ALBUMIN FROM HUMAN URINE

Urine was collected for 24 h from diabetic patients and analyzed for albumin content by both immunoturbidimetry and HPLC. Urine containing  $\sim 50\%$  immunoreactive (e.g.,  $64\text{ mg/L}$ ) and  $50\%$  immunochemically nonreactive (e.g.,  $47\text{ mg/L}$ ) albumin was chosen for further purification. Urine was concentrated  $\sim 5\text{--}10$ -fold by use of a  $50\text{ kDa}$  cutoff membrane (Millipore Corporation). Concentrated urine was purified by an immunosubtraction procedure using cyanogen bromide-activated Sepharose (Sigma-Aldrich Co.) according to the supplier's instructions. The ligand bound to the gel was rabbit anti-human albumin antibody (Dako Cytomation). Unbound albumin (or immunochemically nonreactive albumin) was eluted off the column and was found to have a concentration of  $<6\text{ mg/L}$  by immunoturbidimetry and  $69\text{ mg/L}$  by HPLC.

Affinity-purified immunochemically nonreactive albumin was further purified to remove any remaining contaminants by chromatography on an Agilent 1100 HPLC system (see method described above). Typically, the detected amounts of immunochemically nonreactive albumin from urine were  $70\text{--}80\%$  of the expected values. HPLC-purified immunochemically nonreactive albumin was further concentrated by use of a  $50\text{ kDa}$  cutoff membrane. Purified immunochemically nonreactive albumin was analyzed by native PAGE and reducing (with dithiothreitol) SDS-PAGE. Common urinary proteins, including HSA, transferrin ( $M_r 76\text{ }500$ ),  $\alpha_1$ -acid glycoprotein ( $M_r 44\text{ }100$ ),  $\alpha_1$ -antitrypsin ( $M_r 54\text{ }000$ ), and  $\alpha_2$ -HS-glycoprotein ( $M_r 49\text{ }000$ ), were used as size markers.

#### COMPETITIVE ELISAs

The immunochemically nonreactive albumin preparation was also tested by ELISA for the presence of other common urinary proteins: IgG ( $M_r 160\text{ }000$ ), transferrin,  $\alpha_1$ -acid glycoprotein, and  $\alpha_1$ -antitrypsin. The presence of  $\text{Na}^+/\text{K}^+$ -ATPase  $\beta$ -subunit was also tested by ELISA. IgG, transferrin,  $\alpha_1$ -acid glycoprotein, and adenosine  $5'$ -triphosphatase were purchased from Sigma-Aldrich.  $\alpha_1$ -Antitrypsin was purchased from ICN Biomedicals Inc. Rabbit anti-human IgG (polyclonal to whole molecule), mouse anti-human  $\alpha_1$ -acid glycoprotein (monoclonal), and rabbit anti-human  $\alpha_1$ -antitrypsin (polyclonal) were purchased from Sigma-Aldrich. Rabbit anti-human transferrin (polyclonal) was from Dako Cytomation. Mouse anti-human  $\text{Na}^+/\text{K}^+$ -ATPase  $\beta$ -subunit (monoclonal) was from ABR Affinity Bioreagents. The detection antibody used was alkaline phosphatase-conjugated goat

anti-rabbit IgG (Dako) or goat anti-mouse IgG (Sigma-Aldrich) diluted 1:2000 in assay buffer. The enzyme substrate used was *p*-nitrophenyl phosphate (Sigma-Aldrich). The plate was read at 405 nm.

### Results

#### COMPARISON OF URINARY ALBUMIN ANALYSIS BY HPLC OR RIA AND DENSITOMETRIC ANALYSIS OF ALBUMIN BAND IN ELECTROPHORETIC PROFILES

Urine collected from diabetic patients over 24 h and stored at  $-20^{\circ}\text{C}$  were analyzed by native PAGE. A representative gel is shown in Fig. 1. For three diabetic urines containing relatively large quantities of immunochemically nonreactive albumin, the bands that migrated the same distance on the gel as the albumin calibrator were excised from the gel. These bands were sequenced by LC/MS/MS and were demonstrated to contain only albumin (two of these are shown in Fig. 1). Native PAGE analysis of other plasma protein markers, including  $\alpha_1$ -antitrypsin, transferrin,  $\alpha_1$ -acid glycoprotein, and  $\alpha_2$ -HS-glycoprotein, showed that these proteins migrated differently from urinary albumin (Fig. 2). IgG was too large to enter the native gels.

The results of the native PAGE analysis (Fig. 1) are striking because the major protein found in all of the diabetic urines analyzed was albumin, and because, based on these results, the RIA clearly underestimates the amount of albumin in urine. For example, for the urine sample in the fourth lane from the right in Fig. 1, the RIA would suggest that there is only minimal albumin (3.78 mg/L), whereas from the band intensity and from the HPLC-estimated value (41.3 mg/L), there is clearly a far greater amount of albumin present. To further demonstrate this, we determined the correlation between urinary albumin concentration measured by native PAGE and either HPLC or RIA (Fig. 3A) and found good correlation ( $r^2 = 0.83$ ) between native PAGE and HPLC analysis in

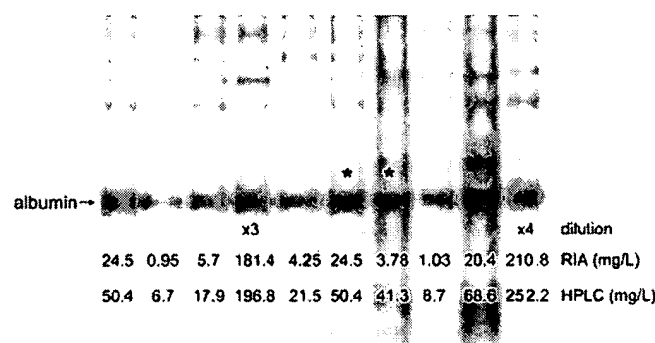


Fig. 1. Native PAGE analysis of diabetic urines containing various amounts of immunoreactive albumin and immunochemically nonreactive albumin.

The amounts of albumin determined by HPLC and RIA are shown, as is the position of the albumin monomer. \*, bands sequenced by LC/MS/MS by the Australian Proteome Analysis Facility. These bands were demonstrated to contain only albumin.

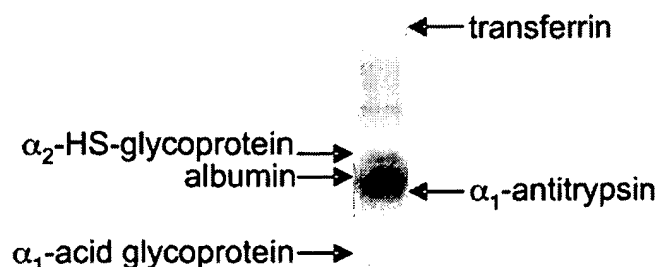


Fig. 2. Native PAGE analysis of HPLC-purified immunochemically non-reactive albumin.

The positions of albumin,  $\alpha_1$ -antitrypsin, transferrin,  $\alpha_1$ -acid glycoprotein and  $\alpha_2$ -HS-glycoprotein are indicated.

spite of the varying ratio of HPLC/RIA concentrations (Fig. 1) but a poor correlation between native PAGE and RIA [ $r^2 = 0.62$  ( $P = 0.0778$  vs the correlation coefficient for native PAGE and HPLC)]. Similar analyses were performed for the same diabetic urines analyzed by reducing SDS-PAGE (Fig. 3B). The results of this analysis were the opposite of those for native PAGE, with a good correlation ( $r^2 = 0.84$ ) between reducing SDS-PAGE and RIA and a poor correlation between reducing SDS-PAGE and HPLC [ $r^2 = 0.65$  ( $P = 0.0895$  vs the correlation coefficient for reducing SDS-PAGE and HPLC)].

These results can be further explained by calculating the relative amount of albumin that was detected (quantity calculated by comparing densitometry values with HSA calibrators run on each gel) by native PAGE and reducing SDS-PAGE compared with that determined by either HPLC or RIA. Under native conditions, the mean (SE) amounts of albumin detected by native PAGE and HPLC were similar [89 (8.1)% of the expected value] compared with that detected by native PAGE and RIA [30 (6.8)% of the expected value]. Under reducing conditions, however, the amounts of albumin detected by reducing SDS-PAGE and RIA were equivalent [98 (5.1)% of the expected value] compared with the amounts detected by reducing SDS-PAGE and HPLC [28 (2.4)%] because, under native conditions, both native PAGE and HPLC detect total albumin (immunoreactive albumin + immunochemically nonreactive albumin). Only immuno-reactive albumin is detected by the RIA; therefore, the amount detected is lower. On the other hand, under reducing conditions, the immunochemically nonreactive albumin fragments into many smaller bands (Fig. 4), and therefore, the amounts detected by reducing SDS-PAGE and RIA become equivalent because both are detecting the immunoreactive albumin species.

Sera from patients with a high urinary content of

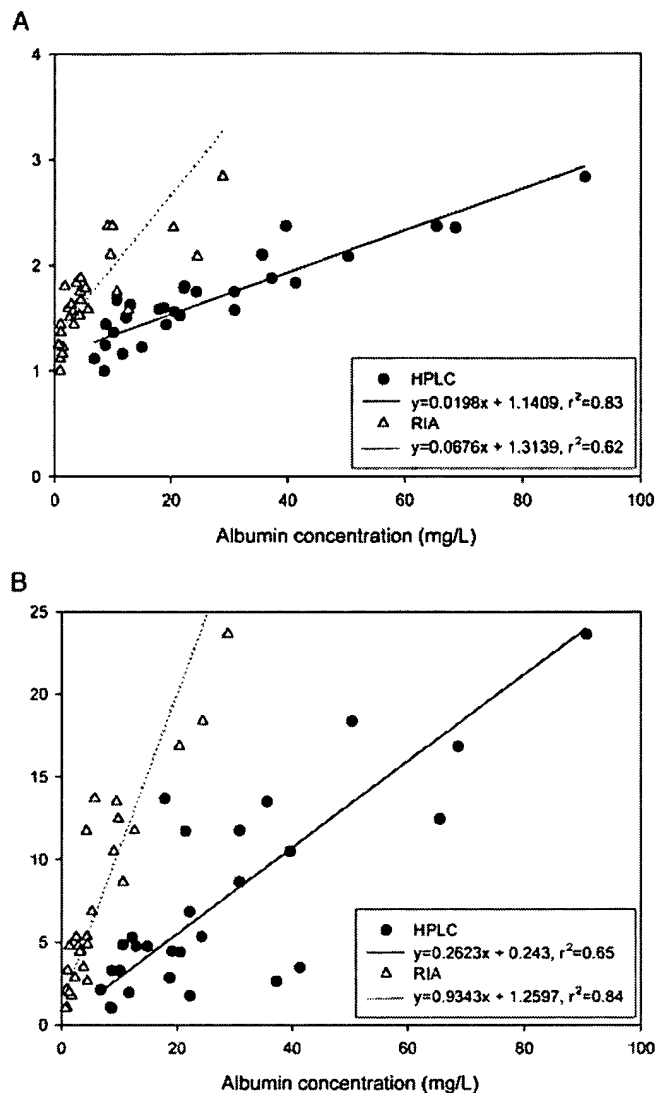


Fig. 3. Relative absorbance of the urinary albumin monomer as determined by computer-aided densitometry after separation by native PAGE (A) or SDS-PAGE (B) compared with urinary albumin concentrations (<100 mg/L) measured by HPLC or RIA.

●, HPLC (mg/L); ▲, RIA (mg/L). Passing-Bablok analysis lines and correlation coefficients are also shown for relative absorbance vs HPLC (solid line) and relative absorbance vs RIA (dashed line).

immunochemically nonreactive albumin were also analyzed by reducing SDS-PAGE (results not shown). These samples did not contain any of the distinctive bands of immunochemically nonreactive albumin when analyzed under these conditions, indicating that this species of albumin is formed during renal passage and is not present in the blood.

In a separate experiment, fresh (unfrozen) urines collected from diabetic patients were also analyzed by native PAGE and HPLC and then reanalyzed after freezing at  $-20$  and  $-80^\circ\text{C}$  for 24 h, 1 week, and 2 months. None of the material present in the fresh urine was lost at either  $-20$  or  $-80^\circ\text{C}$ , nor was extra material produced, as

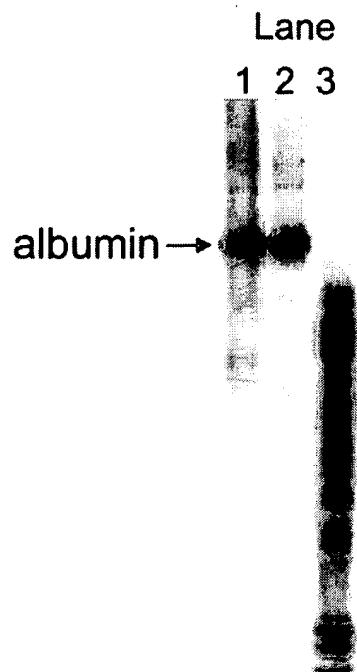


Fig. 4. Electrophoretic analysis of diabetic urine and HPLC-purified immunochemically nonreactive albumin.

Lane 1, undiluted diabetic urine analyzed by native PAGE; lane 2, HPLC-purified immunochemically nonreactive albumin analyzed by native PAGE; lane 3, HPLC-purified immunochemically nonreactive albumin analyzed by reducing SDS-PAGE.

detected by HPLC or native PAGE after freezing (results not shown). In addition, there was no loss or increase in immunochemically nonreactive albumin in diabetic urine after six freeze/thaw cycles (mean HPLC value of 20.6 mg/L for fresh samples; mean value of 21.8 mg/L in samples after six freeze/thaw cycles).

#### ISOLATION OF IMMUNOCHEMICALLY NONREACTIVE ALBUMIN

The HPLC profiles obtained at various stages in the isolation of immunochemically nonreactive albumin from diabetic urine are shown in Fig. 5. The elution profile of HSA is shown in Fig. 5A. Both the monomer (retention time, 9.469 min) and dimer can be seen. In a typical urine profile from a diabetic patient (Fig. 5B), the albumin monomer eluted at 9.474 min. The same urine after concentration through a 50 kDa cutoff membrane is shown in Fig. 5C, and the elution profile of concentrated urine after immunosubtraction using anti-albumin antibodies to remove all immunoreactive albumin is shown in Fig. 5D. At this stage, the preparation was found to contain <6 mg/L albumin by immunoturbidimetry but 69 mg/L albumin by HPLC (retention time, 9.496 min). The preparation was further purified by HPLC fractionation (Fig. 5E). After HPLC fractionation, the preparation eluted as a single peak with a retention time similar to that of HSA and also migrated as a single band under native conditions (Fig. 4, lane 2). This preparation was further concentrated to 0.8 g/L by HPLC but was still found to

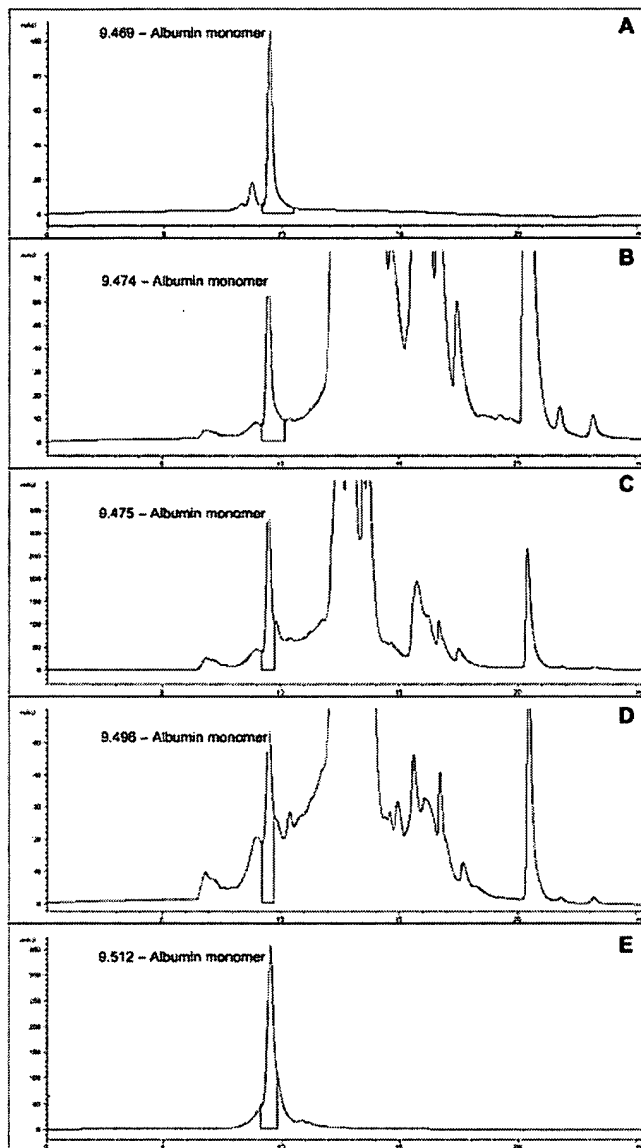


Fig. 5. HPLC profiles obtained at different stages in the isolation of immunochemically nonreactive albumin.

(A), HSA; (B), undiluted diabetic urine; (C), urine after concentration through a 50 kDa molecular cutoff membrane; (D), concentrated urine after affinity purification using anti-albumin antibodies; (E), affinity-purified urine after further purification by HPLC fractionation. The x axes show the elution times in minutes, and the y axes show the absorbance units (mAU) at 214 nm. The retention time for albumin monomer is indicated in each panel.

contain no immunoreactive albumin by both immunoturbidimetry and RIA.

#### ANALYSIS OF IMMUNOCHEMICALLY NONREACTIVE ALBUMIN BY ELISA

The immunochemically nonreactive albumin preparation (0.8 g/L) was analyzed by ELISA for the presence of other proteins commonly found in urine, including IgG, transferrin,  $\alpha_1$ -acid glycoprotein,  $\alpha_1$ -antitrypsin, and  $\text{Na}^+/\text{K}^+$ -ATPase  $\beta$ -subunit. None of the proteins tested was present at a concentration exceeding 1% in the immuno-

chemically nonreactive albumin preparation. The amounts of contamination were most likely less than those indicated because the concentrations of these proteins were below the detection limits of the ELISAs and could not be accurately measured. There was also no immunoreactive albumin present as tested by both RIA and immunoturbidimetry. In addition, <1% Tamm-Horsfall glycoprotein ( $M_r$  80 000–90 000) was detected in the preparation by capillary electrophoresis (5).

#### Discussion

In this study we isolated a species of albumin from urines collected from several diabetic patients that is not detected by conventional antibodies generated against native serum albumin. We named this molecule immunochemically nonreactive albumin. The molecule appears to be the same size as HSA because it elutes at the same position in size-exclusion HPLC and native PAGE. To ensure that the immunochemically nonreactive albumin preparation did not contain other common urinary proteins of similar size to albumin, we tested the preparation for the presence of IgG, transferrin,  $\alpha_1$ -acid glycoprotein,  $\alpha_1$ -antitrypsin,  $\text{Na}^+/\text{K}^+$ -ATPase  $\beta$ -subunit, and  $\alpha_2$ -HS-glycoprotein. There was <1% contamination by any of these other proteins. Similarly, these possible contaminants did not migrate at the same position as the immunochemically nonreactive albumin in native PAGE. In fact, given that the measured amount the protein was 70–80% of the expected and the much smaller amounts of other proteins found in urine compared with albumin (6), it is highly unlikely that we could have isolated such large quantities of any protein other than albumin. Similarly, we have previously analyzed diabetic urines containing a significant amount of immunochemically nonreactive albumin by two-dimensional electrophoresis (2) and found by sequencing that all of the major bands were HSA, demonstrating that albumin is the major urinary protein (2).

Some investigators have described Tamm-Horsfall glycoprotein as the most abundant protein in mammalian urine (7,8), and the reported effects of diabetes on Tamm-Horsfall glycoprotein excretion vary (9). Using capillary electrophoresis, we have been unable to detect any significant quantities of Tamm-Horsfall glycoprotein in the immunochemically nonreactive albumin preparation, nor have we detected this glycoprotein in any of the fresh or frozen diabetic or control urines, using native PAGE or HPLC. In fact, none of the material present in the fresh urine was lost nor was extra material produced, as detected by both of these techniques, after freezing. We may not have observed the presence of Tamm-Horsfall glycoprotein using the techniques in this study because it has a tendency to become gelatinous and aggregate when the sodium chloride concentration of the sample is close to 100 mmol/L or calcium chloride is 1 mmol/L (10).

Under reducing SDS-PAGE conditions, there was a major loss of immunochemically nonreactive albumin in

both undiluted urine and purified immunochemically nonreactive albumin preparations. These results are consistent with the interpretation that immunochemically nonreactive albumin is a composite of peptide chains resulting from limited proteolytic digestion and are held together by disulfide bonds and noncovalent interpeptide chain bonding. It is probable that immunochemically nonreactive albumin is formed as a result of renal passage, although we cannot eliminate the possibility that it is present in low concentrations in blood and that its removal is facilitated by renal clearance. It is of interest that a similar molecule was proposed by Yagame et al. (11), although they demonstrated the presence of albumin fragments by reducing SDS-PAGE and did not demonstrate that the molecule ever existed in an intact form. They did demonstrate the prevalence of relatively large albumin fragments in diabetic urines compared with control urines.

Conventional antibodies generated against native serum albumin do not recognize modified intact urinary albumin (12–14). In fact, we have tested in excess of 30 different polyclonal and monoclonal albumin antibodies and have none that detect immunochemically nonreactive albumin. These antibodies have been raised against serum albumin and not urinary albumin. It is clear that immunoassays measure an active epitope in the urine; however, this epitope may not be associated exclusively with intact albumin (12–14). The immunochemically nonreactive nature of this albumin molecule may be attributable to the fact that the epitope is altered by a conformational change as a result of incomplete processing by the lysosomal pathway, which is compromised in diabetes but not in healthy individuals (12,13), or to the attachment of ligands such as glucose or fatty acids, which are increased in diabetes.

We have recently shown in both diabetic rats and in diabetic patients that the progression to microalbuminuria is accompanied by an increase in the concentration of immunochemically nonreactive albumin. In fact, for patients with type 1 or type 2 diabetes, detection of this albumin species by HPLC can predict the onset of persistent albuminuria 3.9 and 2.4 years earlier, respectively, than can detection of immunoreactive albumin by conventional immunochemistry-based tests (3), with similar results found in peripheral vascular disease (4). Isolation and partial characterization of immunochemically nonreactive albumin may therefore be important for the early detection of the kidney disease associated with diabetes and possibly of other albuminuric conditions, such as those associated with hypertension and cardiovascular disease (15,16).

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## References

- Greive KA, Eppel GA, Reeve S, Smith I, Jerums G, Comper WD. Immuno-unreactive albumin excretion increases in streptozotocin diabetic rats. *Am J Kidney Dis* 2001;38:144–52.
- Comper WD, Osicka TM, Jerums G. High prevalence of immuno-unreactive intact albumin in the urine of diabetic patients. *Am J Kidney Dis* 2003;41:336–42.
- Comper WD, Osicka TM, Clark M, MacIsaac RJ, Jerums G. Earlier detection of microalbuminuria in diabetic patients using a new urinary albumin assay. *Kidney Int* 2004;65:1850–5.
- Brinkman JW, Bakker SJL, Gansevoort RT, Hillege HL, Kema IP, Gans ROB, et al. Which method for quantifying urinary albumin excretion gives what outcome? A comparison of immunonephelometry with high-performance liquid chromatography. *Kidney Int* 2004;in press.
- Jenkins MA. Clinical application of capillary electrophoresis to unconcentrated human urine proteins. *Electrophoresis* 1997;18:1842–6.
- Aronoff SL, Schnider S, Smeltzer J, Mackay W, Tchou P, Rushforth N, et al. Urinary excretion and renal clearance of specific plasma proteins in diabetes of short and long duration. *Diabetes* 1981;30:656–63.
- Fletcher AP, Neuberger A, Ratcliffe WA. Tamm-Horsfall urinary glycoprotein. The chemical composition. *Biochem J* 1970;120:417–24.
- Serafini-Cessi F, Malagolini N, Cavallone D. Tamm-Horsfall glycoprotein: biology and clinical relevance. *Am J Kidney Dis* 2003;42:658–76.
- Chakraborty J, Below AA, Solaiman D. Tamm-Horsfall protein in patients with kidney damage and diabetes. *Urol Res* 2004;32:79–83.
- Stevenson FK, Cleave AJ, Kent PW. The effect of ions on the viscometric and ultracentrifugal behaviour of Tamm-Horsfall glycoprotein. *Biochim Biophys Acta* 1971;236:59–66.
- Yagame M, Suzuki D, Jinde K, Yano N, Naka R, Abe Y, et al. Urinary albumin fragments as a new clinical parameter for the early detection of diabetic nephropathy. *Intern Med* 1995;34:463–8.
- Burne MJ, Panagiotopoulos S, Jerums G, Comper WD. Alterations in the renal degradation of albumin in early experimental diabetes: a new factor in the mechanism of albuminuria. *Clin Sci* 1998;95:67–72.
- Osicka TM, Houlihan CA, Chan JG, Jerums G, Comper WD. Albuminuria in patients with type 1 diabetes is directly linked to changes in the lysosome-mediated degradation of albumin during renal passage. *Diabetes* 2000;49:1579–84.
- Eppel GA, Nagy S, Jenkins MA, Tudball RN, Daskalakis M, Balazs NDH, et al. Variability of standard clinical protein assays in the analysis of a model urine solution of fragmented albumin. *Clin Biochem* 2000;33:487–94.
- Heart Outcomes Prevention Evaluation (HOPE) Study Investigators. Effects of ramipril on cardiovascular and microvascular outcomes in people with diabetes mellitus: results of the HOPE study and MICRO-HOPE substudy. *Lancet* 2000;355:253–9.
- Mogensen CE. Microalbuminuria and hypertension with focus on type 1 and type 2 diabetes. *J Intern Med* 2003;254:45–66.

# STEDMAN'S

## Medical Dictionary

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radiation or medical physics, the number of disintegrations per second of a radionuclide. Radioactivity. Unit (SI): becquerel. [L. *absorptio*, fr. *absorbeo*, to swallow]

**cutaneous a.**, SYN percutaneous a.

**disjunctive a.**, a. of living tissue in immediate relation with a necrosed part, producing a line of demarcation.

**electron resonance a.**, SEE electron spin resonance.

**external a.**, the a. of substances through skin, mucocutaneous surfaces, or mucous membranes.

**interstitial a.**, the removal of water or of substances in the interstitial fluid by the lymphatics.

**parenteral a.**, a. by any route other than the alimentary tract.

**pathologic a.**, parenteral a. of any excremental or pathologic material into the bloodstream, e.g., pus, urine, bile, etc.

**percutaneous a.**, the a. of drugs, allergens, and other substances through unbroken skin. SYN cutaneous a.

**photoelectric a.**, interaction of an x-ray photon with matter in which the incident photon is completely absorbed, giving up all its energy by displacing an outer shell electron.

**ab-sorp-tive** (ab-sōrp'tiv). SYN absorbent (1).

**ab-sorp-tiv-i-ty** (a) (ab-sōrp-tiv'i-tē). 1. SYN specific absorption coefficient. 2. SYN molar absorption coefficient.

**molar a.**, SYN molar absorption coefficient.

**ab-sti-nence** (ab'sti-nens). Refraining from the use of certain articles of diet, alcoholic beverages, illegal drugs, or from sexual intercourse. [L. *abs-tineo*, to hold back, fr. *teneo*, to hold]

**ab-tract** (ab'strakt). 1. A preparation made by evaporating a fluid extract to a powder and triturating with milk sugar. 2. A condensation or summary of a scientific or literary article or address. [L. *ab-straho*, pp. -tractus, to draw away]

**ab-strac-tion** (ab-strak'shūn). 1. Distillation or separation of the volatile constituents of a substance. 2. Exclusive mental concentration. 3. The making of an abstract from the crude drug. 4. Malocclusion in which the teeth or associated structures are lower than their normal occlusal plane. SEE ALSO odontoptosis. 5. The process of selecting a certain aspect of a concept from the whole. [L. *abs-traho*, pp. -tractus, to draw away]

**ab-stric-tion** (ab-strik'shūn). In fungi, the formation of asexual spores by cutting off portions of the sporophore through the growth of dividing partitions. [L. *ab-*, from, + *stric-tura*, a contraction]

**ab-ter-mi-nal** (ab-ter'mi-nāl). In a direction away from the end and toward the center; denoting the course of an electrical current in a muscle. [L. *ab*, from, + *terminus*, end]

**ab-trop-fung** (ab-trop'fūng). A theory that nevus cells are epidermal cells (melanocytes) that proliferate and drop off (migrate) into the dermis. [Ger. *Abtropfung*, trickling down]

**γ-Abu** Abbreviation for γ-aminobutyric acid.

**abu-lia** (ā-bū'lē-ā). 1. Loss or impairment of the ability to perform voluntary actions or to make decisions. 2. Reduction in speech, movement, thought, and emotional reaction; a common result of bilateral frontal lobe disease. SYN aboulia. [G. *a-* priv. + *boulē*, will]

**abu-lic** (ā-bū'lik). Relating to, or suffering from, abulia.

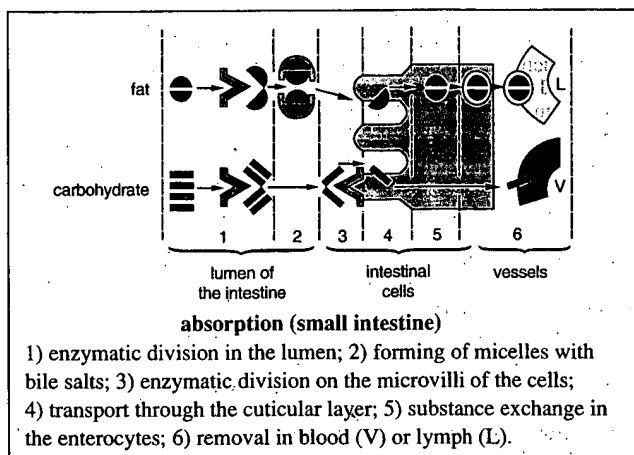
**a-bun-dance** (a'būn-dans). The average number of types of macromolecules (e.g., mRNAs) per cell.

**abuse** (ā-byūs'). 1. Misuse, wrong-use, especially excessive use, of anything. 2. Injurious, harmful, or offensive treatment, as in child a. or sexual a.

**child a.**, the psychological, emotional, and sexual a. of a child, typically by a parent, stepparent, or parent surrogate. SEE domestic violence.

**drug a.**, habitual use of drugs not needed for therapeutic purposes, such as solely to alter one's mood, affect, or state of consciousness, or to affect a body function unnecessarily (as in laxative a.); non-medical use of drugs.

To qualify as drug dependant, a person must use a mood-altering substance daily, for a period of 2-3 weeks or longer. The drug-dependent person must also display certain characteristics, including psychological craving for the substance, symptoms of withdrawal indicating physi-



- absorption (small intestine)**
- 1) enzymatic division in the lumen; 2) forming of micelles with bile salts; 3) enzymatic division on the microvilli of the cells; 4) transport through the cuticular layer; 5) substance exchange in the enterocytes; 6) removal in blood (V) or lymph (L).

ological dependance, and tolerance (need for increased amounts of the drug to reproduce the initial level of response). Behaviorally, the dependent person manifests a reduced ability to function at work or home, and often will appear erratic, moody, or anxious. The use of virtually any drug may lead to dependance. Most commonly, drug dependance involves alcohol, nicotine, cocaine, and the opiates. In addition, some people use psychedelics, marijuana, caffeine, antihistamines, steroids, and solvents to a degree that qualifies as a substance use disorder. Treatment regimens vary in methodology and degree of success.

**elder a.**, the physical or emotional a., including financial exploitation, of an elderly person, by one or more of the individual's children, nursing home caregivers, or others.

**sexual a.**, SEE domestic violence.

**spouse a.**, spousal a., SEE domestic violence.

**substance a.**, maladaptive pattern of drug or alcohol use that may lead to social, occupational, psychological, or physical problems.

**abut-ment** (ā-būt'ment). In dentistry, a natural tooth or implanted tooth substitute, used for the support or anchorage of a fixed or removable prosthesis.

**auxiliary a.**, a tooth other than the one supporting the direct retainer, assisting in the overall support of a removable partial denture.

**intermediate a.**, a natural tooth, or an implanted tooth substitute, without other natural teeth in proximal contact, used along with the mesial and distal a.'s to support a prosthesis; often called a "pier."

**isolated a.**, a lone-standing tooth, or root, used as an a. with edentulous areas mesial and distal to it.

**splinted a.**, the joining of two or more teeth into a rigid unit by means of fixed restorations to form a single a. with multiple roots.

**ABVD** Abbreviation for a chemotherapy regimen of Adriamycin (doxorubicin), bleomycin, vinblastine, and dacarbazine; used to treat neoplastic diseases, such as Hodgkin's disease, shown to be resistant to MOPP therapy.

**ab-volt** (ab'vōlt). The CGS electromagnetic unit of difference of potential equal to  $10^{-8}$  volt. The potential difference between two points such that 1 erg of work will be done when 1 abcoulomb of charge moves from point to point.

**ab-zyme** (ab'zīm). SYN catalytic antibody. [antibody + enzyme]

**AC** Abbreviation for alternating current.

**Ac** Symbol for actinium; acetyl.

**aC** Symbol for arabinosylcytosine.

**a.c.** Abbreviation for L. *ante cibum*, before a meal or *ante cibos*, before meals.

**AC/A** Abbreviation for accommodative convergence-accommodation ratio.

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